SHORT COMMUNICATION

Effect of nanoemulsion on dental unit waterline biofilm

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KEYWORDS
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Abstract Bacterial biofilm in dental unit waterlines (DUWLs) is a widespread problem and poses a potentially significant risk of infection to dental staff and patients. The present study investigates the level and composition of bacterial contamination of dental chair syringe waterlines and investigates the efficacy of a cetylpyridinium chloride-containing nanoemulsion disinfectant in reducing bacterial loads. Waterline biofilms exposed to nanoemulsion for 1 hour, 6 hours, 12 hours, 24 hours, 48 hours, and 72 hours showed high reduction of colonies, and very low counts after 12 hours and 24 hours (67 colony-forming units/mL) were observed. Exposures for 48 hours and 72 hours showed no or few visible colonies (2 colony-forming units/mL). The nanoemulsion employed improves efficacy against microorganisms more than unemulsified components. DNA sequencing showed that the organisms in the waterline biofilm are primarily of soil or water origin. The findings indicate that nanoemulsion effectively disinfects waterlines to consistently meet the American Dental Association (ADA) recommendation.

Introduction
The contamination of dental unit waterlines (DUWLs) is an emerging concern in dentistry because the proportion of elderly and immunocompromised patients seeking dental care is increasing. It is possible that bacteria derived from saliva and plaque from the mouth of one patient can inoculate other patients via dental unit water syringes and hand pieces. A wide variety of commercial waterline cleaning products and systems are available, some of which can be retrofitted to existing DUWLs.1

Nanoemulsions are unique disinfectants with a uniform population of droplets of high energy ranging in diameter from 100 nm to 300 nm.2 Nanoemulsions have broad biocidal efficacy against bacteria, enveloped viruses, and fungi3 by disruption of their outer membranes. We have shown that nanoemulsions are effective against biofilms.4–6

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In addition, cetlypyridinium chloride has biocidal activity by mechanisms distinct from that of nanoemulsions. Therefore, the use of nanoemulsion to control adhesion and formation of biofilm is a logical approach to the problems presented.

Materials and methods

The oil-in-water nanoemulsion was prepared as described in our earlier publication. Particle size was measured using laser light scattering (Dynamic Light Scattering; Brookhaven Instruments, Holtsville, NY, USA).4-6

Five-centimeter pieces of polyurethane tubing (3.2 mm OD; A-dec, Newberg, OR, USA), directly attached to the water syringes in each of three operatories, were cut and rinsed with sterile water to remove planktonic cells. Tubing was aseptically sectioned longitudinally into four equal sections, and then cut horizontally into 5-mm sections. The 5-mm sections were pooled and randomly assigned to the experimental groups.

For live/dead stain biofilm examination, fifty-four 5-mm sections were divided into three groups; Group 1 was immersed in nanoemulsion; Group 2 was immersed in the nanoemulsion ingredients (25% soybean oil, 1% cetlypyridinium chloride, and 10% Triton X-100 in sterile water) in the unemulsified state; and Group 3 was immersed in sterile tap water. At each time point (1 hour, 6 hours, 12 hours, 24 hours, 48 hours, and 72 hours), three tubing sections were removed and stained with L 7012 LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes Inc., Eugene, OR, USA) and Image J1.42q (Wayne Rasband, National Institute of Health, Bethesda, MD, USA). The same set of samples used in our earlier publication. Particle size was measured using laser light scattering (Dynamic Light Scattering; Brookhaven Instruments, Holtsville, NY, USA).4-6

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Percentage dead area ranged from 25 m to 35 m. The biofilm stained with LIVE/DEAD BacLight Bacterial Viability Kit are shown in Fig. 1. The images were analyzed based on the distribution of live (green) and dead (red) areas and the software used were LSM Image Browser (Zeiss, Oberkochen, Germany) and Image J1.42q (Wayne Rasband, National Institute of Health, Bethesda, MD, USA). The same set of samples used for spread plate counting on R2A agar (Difco) plates were used to check viable bacterial counts.

For identification of microorganisms from each DUWL biofilm, organisms were cultured, isolated, and pure-cultured for template DNA preparation. Template DNA and PCR conditions were performed as described previously.8 PCRs were run in a PTC-100 thermocycler (MJ Research, Watertown, MA, USA) using a three-step protocol, which consisted of 30 cycles with an annealing temperature of 58°C and a 1-minute extension time. The PCR products were cleaned using a QIAquick PCR purification kit (Qiagen, Inc., Valencia, CA, USA). Purified templates were sequenced in both directions at the University of Texas Health Science Center at San Antonio Advanced Nucleic Acids Core Facility. A BLASTn search of GenBank at the National Center for Biotechnology Information (NCBI), using a cut-off value of 97% identity, returned the organisms in Table 1 as the top hits.

For statistical analysis, the experiments were performed in triplicate and the means and standard deviations calculated. Statistical significance was determined using two-way ANOVA with replication using Microsoft Excel, with the level of significance (α) set at 0.05.

Results

Microfluidizer emulsification resulted in droplets with a mean diameter of 193 nm.5,6 Waterline biofilms exposed to nanoemulsion for 1 hour, 6 hours, 12 hours, 24 hours, 48 hours, and 72 hours showed high reduction of colonies, and very low counts after 12 hours and 24 hours (67 colony-forming units/mL) were observed. Exposures for 48 hours and 72 hours showed no or few visible colonies (2 colony-forming units/mL). The biofilm stained with LIVE/DEAD BacLight Bacterial Viability Kit were shown in Fig. 1. The nanoemulsion treatment group showed greatest efficacy against tubing biofilm with time. The nanoemulsion ingredients group also showed efficacy against tubing organisms, but to a lesser extent than the emulsified nanoemulsion group, indicating that emulsions have efficacy in excess of their components. Percentage dead area at 48 hours and 72 hours in the nanoemulsion treatment group was 99.8%. Biofilm thicknesses in the three groups ranged from 25 μm to 35 μm.

Table 1  Bacteria identified by DNA sequencing.

<table>
<thead>
<tr>
<th>Identified bacteria</th>
<th>Gram stain</th>
<th>Identified locale</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Methylobacterium</em> sp.*</td>
<td>Negative</td>
<td>Common in soils and on plants</td>
</tr>
<tr>
<td><em>Micrococcus</em> luteus</td>
<td>Positive</td>
<td>Found in soil, dust, water, air, and skin</td>
</tr>
<tr>
<td><em>Sphingomonas</em> sp.</td>
<td>Negative</td>
<td>Found on land and water, plant root systems, and clinical specimens</td>
</tr>
<tr>
<td><em>S. dokdonensis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. natatoria</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Erythromonas ursincola</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. trueperi</em> strain LMG 2142</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. mathurensis</em> strain SM13</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Ralstonia metallidurans</em></td>
<td>Negative</td>
<td>Found in sediments and soils with heavy metal-contaminated environments</td>
</tr>
<tr>
<td><em>Cupriavidus metallidurans</em></td>
<td>Negative</td>
<td>Found in sediments and soils with heavy metal-contaminated environments</td>
</tr>
<tr>
<td><em>Staphylococcus</em></td>
<td>Positive</td>
<td>Skin, a small component of soil microbial flora</td>
</tr>
<tr>
<td><em>S. pasteuri</em> strain ATCC51129</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. pasteuri</em> strain ZA-b3</td>
<td>Negative</td>
<td>Found in freshwater lakes and streams</td>
</tr>
<tr>
<td><em>Caulobacter</em> sp.*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Discussion

Dental professional organizations have affirmed that it is unacceptable to expose patients (especially immune-compromised or medically compromised patients) or dental and support staff to contaminated DUWL output water or aerosols generated during the use of dental instruments connected to DUWLs. At present, a diverse range of commercial DUWL treatment products and systems are marketed to control or eradicate DUWL biofilm.\(^1\)

The application of nanoemulsion is a new and promising innovation.\(^2\) Nanoemulsion has been reported to have

![Table and Figure](image-url)
extensive bactericidal, sporidal, and virucidal effects.\textsuperscript{3} Nanoemulsion at biocidal concentrations is nontoxic to skin, mucous membranes, and the gastrointestinal tract. Because the mechanism of action of nanoemulsion appears to be the nonspecific disruption of bacterial cell membranes, nanoemulsions would not result in the development of resistant strains. Nanoemulsion can be diluted and stored at a broad range of temperatures for up to 2 years. Bacteria within biofilms are notoriously difficult to eradicate with antimicrobials or biocides. We have demonstrated the efficacy of nanoemulsions against biofilms composed of organisms involved in the caries process,\textsuperscript{4–6} and in the present study, against DUWL biofilms.

In the present study, the majority of microbes found are Gram-negative water bacteria of the same varieties that survive in small numbers in municipal water systems.\textsuperscript{9} Our results (Table 1) show primarily Gram-negative soil- or water-borne bacteria. However, Micrococcaceae and \textit{Staphylococcus} are commonly found on skin. Lax hand-washing discipline and careless handling of bottle and feeder tubes can result in contamination of the water systems with enteric or skin organisms. A case of coliform contamination in dental units has been reported.

The significant reduction in live bacteria observed in nanoemulsion-treated DUWL tubing and a dead area of 99.8\% showed that nanoemulsion effectively reduced DUWL communities. The group treated with unemulsified ingredients also showed efficacy against tubing organisms, but to a lesser extent than the emulsified nanoemulsion group, indicating that emulsions have efficacy in excess of their components. The inventor of the nanoemulsion has proposed that during preparation of nanoemulsions under high shear forces in a microfluidizer, the shear energy is stored in the oil droplets, giving them high energy. He further proposes that this energy is passed to bacteria upon fusion of the droplet with the bacteria, disrupting the bacterial membrane.\textsuperscript{3}

The nanoemulsion formulation also contains cetylpyridinium chloride, a quaternary ammonium salt. Cetylpyridinium chloride places a positive surface charge on the nanodroplet by being incorporated as a cosurfactant. Bacteria have a net negative surface charge. In addition, exopolysaccharide chains, which vary in size from 103 kDa to 108 kDa, are usually negatively charged, sometimes neutral or rarely positively charged. Positively charged nanodroplets should have increased potential to interact with the biofilm cells and matrix. Cetylpyridinium chloride has antimicrobial activity of their own, apparently through multiple mechanisms (disruption of intermolecular interactions, cellular membrane, cellular permeability controls, and inducing leakage of cellular contents). Longer exposure times may result in additional breakdowns of intracellular material, which are indicative of autolysis.\textsuperscript{7} In addition, cetylpyridinium chloride has an inhibitory action against fructosyltransferases, extracellular enzymes that synthesize fructans from sucrose, which serve as an extracellular nutrition reservoir for bacteria. We suspect that dead areas found in the unemulsified ingredients column of Fig. 1 result from cetylpyridinium chloride activity.

The separate mechanisms of action of both nanoemulsion and cetylpyridinium chloride may be operating to reduce biofilm formation in DUWLs. In addition, it is possible that the positively charged emulsion remains attached to the biofilm for an extended time.

We conclude that cetylpyridinium chloride-containing nanoemulsion appears to present a feasible means of disinfection of DUWL output water.

**Acknowledgments**

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**References**


